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Alves, Ana Catarina

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Influence of doxorubicin on model cell membrane properties: insights from *in vitro* and *in silico* studies

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Ana Catarina Alves[1], Aniket Magarkar[2,3], Miguel Horta[1], Jose L. F. C. Lima[1], Alex Bunker[3], Cláudia Nunes[1] & Salette Reis[1]

Despite doxorubicin being commonly used in chemotherapy there still remain significant holes in our knowledge regarding its delivery efficacy and an observed resistance mechanism that is postulated to involve the cell membrane. One possible mechanism is the efflux by protein P-gp, which is found predominantly in cholesterol enriched domains. Thereby, a hypothesis for the vulnerability of doxorubicin to efflux through P-gp is its enhanced affinity for the ordered cholesterol rich regions of the plasma membrane. Thus, we have studied doxorubicin's interaction with model membranes in a cholesterol rich, ordered environment and in liquid-disordered cholesterol poor environment. We have combined three separate experimental protocols: UV-Vis spectrophotometry, fluorescence quenching and steady-state anisotropy and computational molecular dynamics modeling. Our results show that the presence of cholesterol induces a change in membrane structure and doesn't impair doxorubicin's membrane partitioning, but reduces drug's influence on membrane fluidity without directly interacting with it. It is thus possible that the resistance mechanism that lowers the efficacy of doxorubicin, results from an increased density in membrane regions where the efflux proteins are present. This work represents a successful approach, combining experimental and computational studies of membrane based systems to unveil the behavior of drugs and candidate drug molecules.

Doxorubicin (as shown in Fig. 1) is one of the most widely prescribed anticancer drugs[1]. The most prevalent opinion is that the anticancer activity of the drug is mainly due to direct interaction with nucleic acids, leading to DNA damage and inhibition of DNA synthesis. This is, however, still the subject of considerable debate[1]; there is also strong evidence suggesting that interaction with cell membranes plays a role in its activity[2]. Clearly, even if the dominant mechanism is the interaction with the nucleic acids, doxorubicin must pass through a variety of other organelles to reach the DNA[3]. First, the drug must pass the selective barrier to entry into the cell that is the cell membrane and, finally, the nuclear membrane. Thereby, interaction with lipid membranes is an unavoidable step in doxorubicin activity, whatever the exact mechanism of action proves to be.

Drug-membrane interactions produce alterations in the physical properties of the cell membrane, as a result of the behavior of the drug within the membrane. How the drug orients in the membrane, which components of the lipid membrane the drug interacts with and what interactions within the membrane the drug disrupts, will all alter the physical properties of the membrane[4]. This can, in turn, lead to changes in cell signaling and the function of membrane proteins, e.g. transporters and ion channels[5]. It was, for example, found that Daunorubicin (a doxorubicin analog) causes alteration of both G-proteins and protein kinase C-associated signaling pathways, through destabilizing the non-lamellar membrane structures that are involved in their location and activity[6,7]. As these proteins participate in fundamental functions of the cell, including proliferation and differentiation, their modulation could be responsible for the antitumor activity of some drugs[6].

Within the membrane, the lipids are heterogeneously distributed into dynamic domains; domains enriched in cholesterol and sphingolipids are thought to form a highly ordered, liquid-ordered phase (l_o), *rafts* that metaphorically float in a liquid-disordered phase (l_d) matrix[8]. The function of several membrane proteins is highly dependent on their association with these domains[9,10]. Among the proteins thought to be associated with lipid

[1]UCIBIO, REQUIMTE, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal. [2]Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Flemingovo nám. 2, 16610, Prague 6, Czech Republic. [3]Faculty of Pharmacy, University of Helsinki, Viikinkaari 5E, Helsinki, 00014, Finland. Ana Catarina Alves and Aniket Magarkar contributed equally to this work. Correspondence and requests for materials should be addressed to C.N. (email: cdnunes@ff.up.pt)

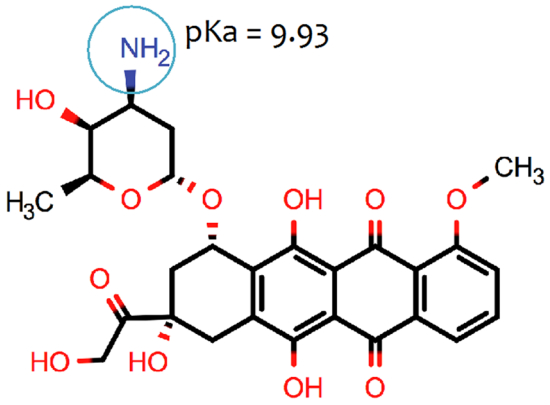

Figure 1. Molecular structure and pKa value of doxorubicin.

rafts[11] is P-glycoprotein (P-gp)[12] that effluxes a variety of hydrophobic, neutral, and positively charged drugs from the cell. P-gp is a component of the normal cellular defense system against xenobiotics[13]. This protein has been shown to be highly sensitive to membrane fluidity; thus, any changes in membrane fluidity will alter the ability of P-gp to efflux drug molecules[11].

Doxorubicin is among the substrates effluxed by P-gp; thus, in this case, this protein plays a key role in drug resistance[14]. What is not so well understood, however, is: 1) how doxorubicin partitions between the l_o rafts and the l_d matrix and 2) how doxorubicin alters the physical properties of the lipid rafts. This has considerable relevance regarding the behavior of doxorubicin as a drug. In fact, the manner in which doxorubicin partitions between the two phases of the plasma membrane will determine its susceptibility to P-gp: the greater the extent to which doxorubicin partitions to the P-gp rich rafts the greater its susceptibility to efflux. Additionally, the effect of the presence of doxorubicin on the structure of the l_o raft can not only effect P-gp function, but also result in the modulation of the physicochemical properties of the membrane itself. This could, in turn, initiate different processes implicated in the anticancer cytotoxic effects of the drug. Several anticancer drugs, in fact, induce modifications in the content of lipid rafts, leading to growth inhibition and activation of tumor cells apoptosis[9].

In light of all of this, we set out to apply a combined protocol of *in vitro* experimental analysis techniques, including UV-Vis spectrophotometry, fluorescence quenching and steady state anisotropy, both using membrane bound fluorescent probes and *in silico* computational modeling, to study the interaction of doxorubicin with the plasma membrane. Properties studied include: 1) effect of doxorubicin on membrane fluidity, 2) distribution of doxorubicin between the l_o and l_d phases and, 3) through computational molecular dynamics modeling, the microscopic details of the membrane-doxorubicin interactions. We show that cholesterol, present in the l_o rafts but not so prevalent in the l_d matrix, alters the behavior of doxorubicin in the membrane without directly interacting with it. This interaction occurs in a fashion that promotes increased location of doxorubicin to the l_o rafts, possibly increasing uptake by the P-gp efflux protein. We propose that this could possibly be a mechanism that contributes to the doxorubicin chemoresistance problem.

Results and Discussion

Doxorubicin Partition Coefficients. The partitioning of drugs into biological membranes plays a significant role in their uptake, transport, bioavailability and distribution[15]. In fact, doxorubicin needs to cross the cell membrane to reach its intracellular targets, for example DNA, and the interaction with lipids can strongly influence the pharmacokinetic and pharmacodynamic properties[15] of the drug. The partition coefficient (Kp), is an important property to determine, as it provides information concerning the distribution of drugs between aqueous and lipid phases, of direct relevance to the pharmacological activity of the drug. The doxorubicin partition coefficient in several model membranes was determined through derivative UV-Vis spectrophotometry. This technique, based on the fact that the spectral characteristics (λ_{max}) of the drug change when it permeates from the aqueous to the lipid medium, allows for the quantification of drug distribution between each phase. Furthermore, it also provides a better resolution of the overlapped bands and eliminates the lipid light scattering interference through the use of the derivative method.

Figure 2(A) shows, as an example, the third-derivative absorption spectra of doxorubicin with different concentrations of liposomes composed of DMPC:SM [8:2] at pH 7.4 and at 37 °C. The use of the third-derivative allowed the elimination of the residual background signal caused by scattering from the liposomes. The best fit of equation 1 to the third-derivative data was achieved at 544 nm and is shown in Fig. 2(B).

The experimental values obtained for the doxorubicin partition coefficient with DMPC:SM and DMPC:SM:Chol (expressed as Kp and logD) are depicted in Table 1.

Traditionally, the partition coefficient is assessed in octanol/water systems. The octanol/water logD of doxorubicin at pH 7.4, obtained using Marvin sketch calculator software (ChemAxon), was 0.02. Thereby, a lack of correlation between the doxorubicin partitioning coefficients obtained in liposome/buffer and octanol/water systems can be observed. Such difference is due to the fact that the biphasic octanol/water system can only account for hydrophobic interactions. Liposomes, on the other hand, provide an anisotropic environment that can better mimic membranes, which are composed by amphiphilic phospholipids and can, therefore, establish electrostatic

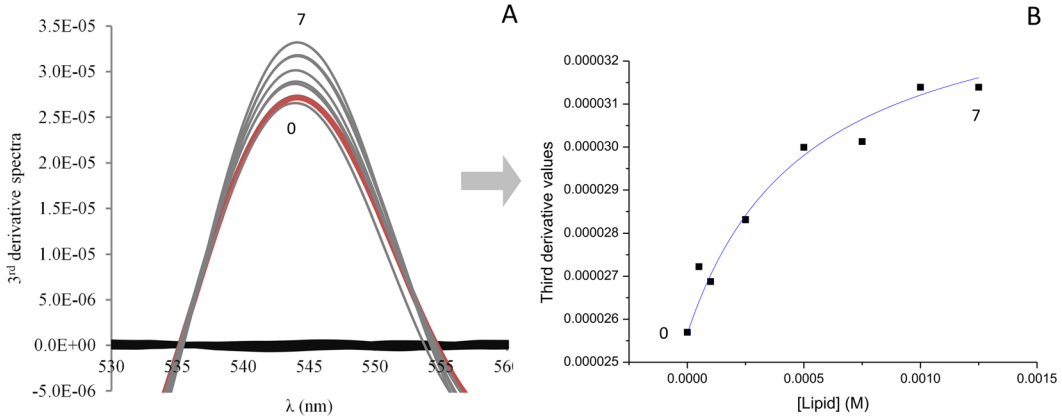

Figure 2. Third-derivative absorption spectra (**A**) of doxorubicin (40 μM) (red line, 0) alone, incubated in DMPC:SM model membrane at 37 °C with increasing lipid concentration (7 represents the maximum lipid concentration) and the model membrane without drug (black lines). (**B**) represents the best fitting curve to experimental third-derivative spectrophotometric data (DT vs. [L]) using a nonlinear regression method at a wavelength of 544 nm.

Model	Kp	Experimental logD
DMPC:SM	8195 ± 406	3.91 ± 0.02
DMPC:SM:Chol	8707 ± 523	3.94 ± 0.03

Table 1. Partition Coefficients (expressed as Kp and logD) of doxorubicin in DMPC:SM [8:2] and DMPC:SM:Chol [7:1.5:1.5] at pH 7.4 and 37 °C[a]. [a]All values represent the mean ± standard deviation (n = 3). *$p < 0.05$ statistically different from the other model.

and hydrophobic interactions. In addition, the pK_a value (9.93), calculated using Marvin sketch calculator software, predict that nearly 80% of doxorubicin molecules are in the cationic form at pH 7.4, providing evidence that not only hydrophobic intermolecular forces drive the doxorubicin's partitioning. In fact, the octanol/water partition coefficient value is much smaller than that calculated by the liposome/buffer system, since it only reflects the hydrophobic interactions of a small number of molecules that are in the neutral form at pH 7.4. Hence, the use of a liposomes/buffer system allows one to obtain more realistic information regarding doxorubicin lipophilicity and, therefore, it's *in vivo* membrane partitioning.

Furthermore, the gathered experimental results reveal that doxorubicin exhibits a similar membrane partitioning with both DMPC:SM and DMPC:SM:Chol liposomes. This suggests that the positively charged doxorubicin interacts with the phospholipid polar head groups of both models and that the presence of Chol does not impair such partitioning.

Drug location studies using different fluorescent probes. The location of doxorubicin in the membrane was tracked through fluorescence quenching of membrane bound probes (TMA-DPH and DPH), since this technique provides a measure of the accessibility of the drug to each probe[16]. Such knowledge is only possible to obtain due to the fact that the fluorophore position in the membrane is well defined and documented. Therefore, while DPH is deeply incorporated in the hydrophobic regions of the lipid bilayer[17], TMA-DPH is reported to be anchored in the polar head groups region of phospholipids due to its charged group[18]. According to this, in the current study the steady-state fluorescence intensities and lifetimes were measured in liposomes labeled with TMA-DPH and DPH probes. The theory and equations behind this technique are presented in the supporting information.

In Fig. 3, the Stern-Volmer plots of $I_0/I-1$ and $\tau_0/\tau-1$ as a function of doxorubicin membrane concentration are shown, for the DMPC:SM [8:2] model.

According to the description of the quenching behavior (see supporting information), the quenching parameter values, namely the dynamic constant (K_D), the static constant (K_S) and the Stern–Volmer constant (K_{SV}), obtained from this technique are depicted in Table 2.

From the analysis of Table 2, it is possible to conclude that the quenching process, in all mimetic model systems studied and probes used, results from both dynamic and static interactions. This behavior indicates that doxorubicin must diffuse into the fluorophore during the lifetime of the excited state; however, it can also form a non-fluorescent complex with the probe. According to the results, doxorubicin was able to quench both probes, DPH and TMA-DPH. Nevertheless, the K_{SV} values demonstrate that the decrease of the DPH fluorescence was more pronounced than for TMA-DPH in the membrane model composed of DMPC:SM [8:2]. This suggests that doxorubicin interacts with the lipid head groups of the phospholipids, but the drug's dihydroanthraquinone residue may also interact with the lipid fatty acid chains through hydrophobic interactions. For the bilayer comprised by DMPC:SM:Chol [7:1.5:1.5] similar values of K_{SV} were obtained for both fluorescent probes, which means

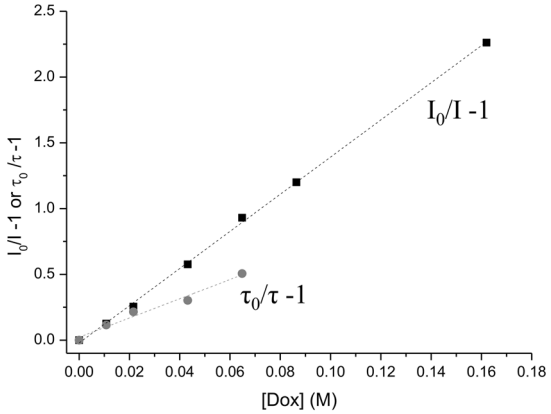

Figure 3. Stern–Volmer plots of the probe DPH in DMPC:SM [8:2] model at pH 7.4 and 37 °C with increasing doxorubicin concentrations: square symbols (■) represent the Stern–Volmer plot obtained by steady-state fluorescence measurements ($I_0/I-1$) and circle symbols (●) represent the Stern–Volmer plot obtained by lifetime fluorescence measurements ($\tau_0/\tau-1$).

Model	Probe	K_D (M^{-1})	K_S (M^{-1})	K_{SV} (M^{-1})
DMPC:SM	DPH	7.4* ± 0.2	6.4 ± 0.3	13.8* ± 0.4
	TMA-DPH	6* ± 1	6.0 ± 0.3	11* ± 1
DMPC:SM:Chol	DPH	3.2* ± 0.4	3.2* ± 0.2	6.4 ± 0.2
	TMA-DPH	2.5* ± 0.2	4.2* ± 0.2	6.6 ± 0.1

Table 2. Fluorescence quenching parameters (K_D, dynamic constant; K_S, static constant; K_{SV}, Stern–Volmer constant) determined from the quenching of DPH and TMA-DPH by doxorubicin in the different mimetic models at pH 7.4 and 37 °C[a]. [a]All values represent the mean ± standard deviation (n = 3). *$p < 0.05$ statistically different from the other probe, for each fluorescence quenching parameter.

that doxorubicin has an identical accessibility to DPH and TMA-DPH in the model membranes with cholesterol present.

Effect of Doxorubicin in membrane fluidity. Since doxorubicin interacts with membranes in order to exert its therapeutic action, it is important to understand how the compound can influence the physicochemical state of the phospholipid bilayer in order to explain its pharmacological action. Furthermore, alterations of membrane fluidity can severely affect the cell's functional properties that might be correlated with the drug's mechanism of action. In this context, membrane fluidity studies were performed by steady-state anisotropy[19] using fluorescent probes (DPH and TMA-DPH). The ability of doxorubicin to disturb the membrane structure in different regions can be assessed since these two probes report the microfluidity of those sites[20]. Modifications in membrane fluidity can be detected by changes in anisotropy, which reflects perturbations in the probe's rotational movement caused by the changes in the stiffness of its surrounding matrix[21].

The effect of temperature on the DPH and TMA-DPH fluorescence anisotropy of the different model membranes without doxorubicin is presented in Fig. 4. The results demonstrate that the anisotropy values in both model membranes decrease with increasing temperature to a greater extent in the acyl chain region (given by the probe DPH) than in the phospholipid head region (given by the TMA-DPH probe). In fact, it is well documented that the membrane is characterized by a fluidity gradient from the aqueous interface to the bilayer interior, where the acyl chain end shows increased disorder. Also, it is known that the presence of SM and Chol in membranes promotes the formation and maintenance of specific domains in the liquid-ordered phase, more condensed and organized[22], which results in higher anisotropy values.

Figure 5 shows the influence of doxorubicin on the DPH and TMA-DPH fluorescence anisotropy as a function of temperature for each model system studied. Regarding the model membrane composed of DMPC:SM two distinct doxorubicin behaviors can be observed in the different regions of the membrane. Namely, doxorubicin increased the membrane fluidity of the phospholipid's acyl chains, in a more ordered phase of the bilayer, and decreased the membrane microviscosity, in a more fluid phase of the bilayer. This represents a similar behavior as Chol possesses when in incorporated in the membrane, helping to prevent the membrane from becoming either too fluid or too rigid, due to the ability of cholesterol to cause disordering of gel-phase lipids while still maintaining a high degree of orientational ordering. At the same time, DOX decreased the membrane microviscosity in the phospholipid's head groups. These findings are, once again, consistent with the previous results of partition and location, where it has been observed that the anticancer drug establishes electrostatic interactions and hydrogen bonds with the negative phosphate group and hydrophobic interactions with the hydrocarbon chains of the phospholipids.

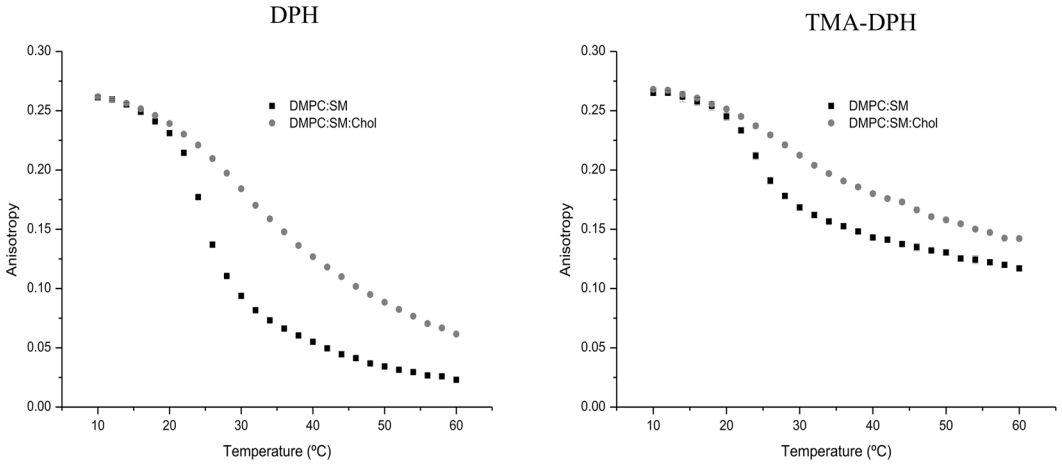

Figure 4. Steady-state anisotropy of DPH and TMA-DPH as a function of temperature in DMPC:SM [8:2] and DMPC:SM:Chol [7:1.5:1.5] membrane models. Results present the mean of at least three independent assays.

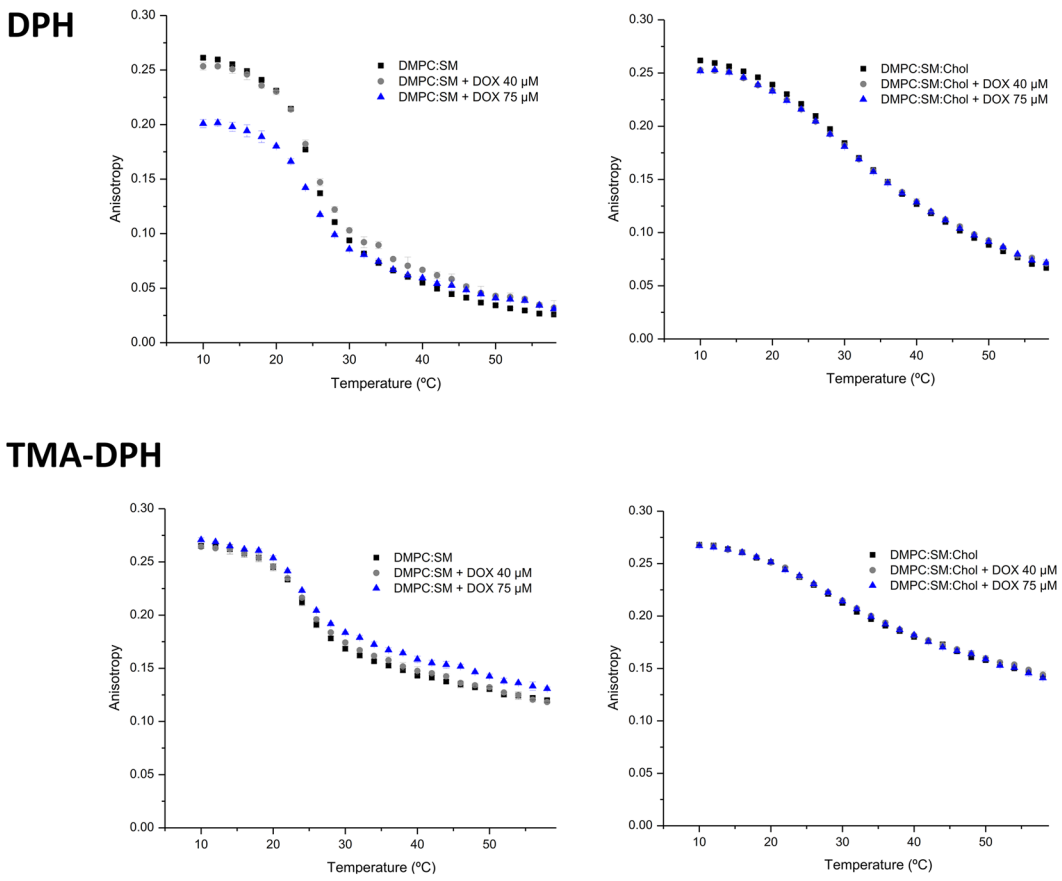

Figure 5. Steady-state anisotropy of DPH and TMA-DPH as a function of temperature in each mimetic model, in the absence (■), and in the presence of doxorubicin 40 μM (●) and 75 μM (▲). Results present the mean of at least three independent assays.

On the other hand, it can be observed that doxorubicin did not produced any alterations in the membrane fluidity of DMPC:SM:Chol model along the bilayer. This fact can be correlated with the disposition of the molecules within the phospholipid membrane. Doxorubicin is a molecule that possesses an aromatic ring wherein its planar structure bears resemblance to the structure of cholesterol. According to the location studies, doxorubicin has an identical accessibility to both DPH and TMA-DPH probes. Considering that the presence of Chol reduces the membrane fluidity, it is possible that doxorubicin does not cause significant alteration in the overall fluidity in both superficial and deeper regions of phospholipids. Furthermore, due to interactions between NH_3^+

of doxorubicin and the phosphate group in the polar head of the phospholipids, the further access of doxorubicin into the membrane might be impaired.

The presence of SM together with Chol form within the membrane, liquid-ordered domains that are characterized by a low density and rigid environment, as shown through anisotropy measurements. It has been demonstrated by several structure–activity studies that hydrophobicity and the presence of planar aromatic regions, as in the case of doxorubicin, favor the drug-P-gp interaction.[28] Furthermore, it was also reported that increase of membrane fluidity can lead to an inhibition of P-gp function.[24] Since our results demonstrate that doxorubicin did not cause any changes in the fluidity of the DMPC:SM:Chol model membrane and that the drug's binding sites on the P-gp are via the lipid membrane,[25] it is possible to correlate the gathered outcomes with the resistance problem associated with doxorubicin administration.

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We used MD simulation with all atom resolution to study the partitioning of doxorubicin into the DMPC:SM and DMPC:SM:Chol membrane bilayers. In all simulations, doxorubicin molecules were placed in the solvent at least 1.5 nm away from the lipid headgroups. The z-coordinate of the center of mass of doxorubicin molecule and z-coordinate of the center of mass of phosphate group vs time is shown in Fig. 6A and B. This analysis shows that the doxorubicin molecule partitions closer to the lipid-water interface in the presence of cholesterol, in good agreement with the derivative spectrophotometry studies. Furthermore, the angle of distribution of the doxorubicin molecule with the membrane normal (shown in red in Fig. 6C) shows that the orientation of the anticancer drug in the presence of cholesterol is significantly altered. As seen in Fig. 6C and Fig. S2A, in the DMPC:SM membrane bilayer, the doxorubicin cyclic group (dihydroanthraquinone residue) is perpendicular to the membrane normal; however for the DMPC:SM:Chol membrane, the presence of cholesterol increases the degree of membrane ordering, resulting in an orientation change such that the average angle of doxorubicin to the membrane normal becomes 22 degrees. Such a difference in the doxorubicin orientation, concerning the two model membranes, might help to explain why alterations in membrane fluidity are more pronounced in the DMPC:SM bilayer. Furthermore, the increase in membrane ordering that results from the presence of cholesterol is traduced by an increase in membrane thickness, as shown in the Fig. S2B. Also, the addition of cholesterol decreases the number of non-bonded contacts of lipids and cholesterol with doxorubicin as shown in Fig. S3. However, in the presence of cholesterol, the number of hydrogen bonds between doxorubicin and both the lipids and cholesterol increases, as presented in Figure S4A and B. Also in Figure S4, we display the specific groups of doxorubicin involved in hydrogen bonding with lipids and both cholesterol (A) and water (B).

The high partitioning of doxorubicin with the two models can be explained by its ability to interact with the lipid tails as demonstrated in Figure S3 and to form hydrogen bonds with lipid headgroups (Figure S4).

In the previous simulation studies by Yacoub et al.[26] it was demonstrated that the doxorubicin molecule has a significant effect on lipid ordering in the DPPC membrane bilayer. In this light, to investigate the effect of the insertion of a single doxorubicin molecule into the model membranes chosen for this study, we calculated the deuterium order parameter for the sn-2 chain of the DMPC molecule in both presence and absence of the doxorubicin molecule. The deuterium order parameter, S_{CD},[27] is a property of the bilayer that provides information concerning the level of lipid chain ordering. It can be obtained accurately from NMR experiments and is defined as follows:

$$S_{CD} = \langle 3/2 \cos^2 \theta_i - 1/2 \rangle \quad (1)$$

where θ_i is the angle between the C–D bond (C–H in simulations) of the i^{th} carbon atom and the bilayer normal. The angular brackets denote averaging over time and over relevant C–D bonds in the bilayer.

As shown in Fig. 7, we see that the change in the deuterium order parameter and thus, the degree of lipid chain ordering, that results from the presence of the doxorubicin molecule, is more pronounced for the DMPC:SM membrane than for the DMPC:SM:Chol membrane. This observation is in agreement with the fluidity measurements obtained from fluorescence anisotropy, as shown in Fig. 5.

We thus observed that, as a result of the presence of cholesterol: 1) the orientation of the doxorubicin molecule in the membrane bilayer is altered, 2) the number hydrogen bonds between Doxorubicin and the membrane lipids is increased and 3) the extent of non-bonded interactions is decreased (Fig. S3). Despite of this marked effect, the extent of direct interaction between cholesterol and doxorubicin was found to be relatively insignificant, as seen in Fig. 8. This might be explained by the fact that the OH group of cholesterol is masked by the polar